Cultrex® Angiogenesis System

Endothelial Cell Tube Formation Assay Troubleshooting

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Angiogenesis is the process whereby endothelial cells build new blood vessels from existing vasculature.

**Physiologic Angiogenesis**
- Embryonic development
- Wound healing
- Reproduction

**Pathological Angiogenesis**
- Excessive
- Insufficient

**Tumor growth and metastasis**
- Rheumatoid arthritis
- Diabetic retinopathy
- Inflammatory diseases
- Atherosclerosis

**Chronic wound**
- Coronary artery disease
- Stroke
- Non-union fracture
In vitro angiogenesis assays:

- Proliferation of endothelial cells
- “Wound healing” migration
- Cell invasion/migration

In vivo angiogenesis assays:

- Directed in vivo angiogenesis assay (DIVAA)
- Rat aortic ring
- Chick chorioallantoic membrane (CAM)
- Corneal micropocket
- Basement membrane subcutaneous “plug”
Tube Formation Assay based on the ability of endothelial cells to form three-dimensional capillary-like tubular structures when cultured on a gel of basement membrane extract (BME)

Tube Formation Assay represents a simple, quantitative, reliable, and powerful model for studying inhibitors and activators of angiogenesis
Add 50 µl of RGF BME per well of 96-well plate

Incubate plate for 30 min at 37°C allowing RGF BME to gel

Trypsinize, harvest and count HUVECs

Incubate plate for 4-20 hours at 37°C until tube network formed

Add diluted HUVECs (15,000 cells in 100 µl) per well on gelled RGF BME

Dilute HUVECs in Endothelial Medium ± angiogenic mediators and inhibitors

Imaging

Tube Formation Assay
Human Umbilical Vein Endothelial Cells (HUVECs were seeded on RGF BME gel at 4,800 cells/cm² and incubated in the Endothelial Growth Medium-2 (EGM-2).
Variables Affecting Tube Formation Assay

- **Extracellular matrix environment:**
  - Type of the Basement Membrane extract (Normal BME or RGF BME)
  - Thickness/density of BME gel

- **Conditions of the cell culture (number of passages, % confluency, cell “health”)

- **Induction of angiogenesis (growth factors and FBS)

- **Cell seeding density (cells/cm²)

- **Length of the assay (hours)**
Cell seeding density affects formation of tubular-like network

Human Umbilical Vein Endothelial Cells (HUVECs) were seeded on RGF BME gel at various concentrations and incubated for 16 hours in Endothelial Growth Medium-2 (EGM-2;). *NOTE* between 4,800 and 6,400 cells/cm² is optimal.
Cell culture conditions (cell “health”)

- Primary cells lose tissue-specific characteristics after several passages (HUVECs after passage 6)

- Cells must be allowed to recover from cryogenic freezing. Subsequent passages select for healthy, dividing cells (usually passage 2 or three)

- Cells must not be over-confluent, the optimal density of cell culture for tube formation assay is 70-80%.
Passage number after thawing effects tube formation

Human Umbilical Vein Endothelial Cells (HUVECs at passages 0 and 2 were plated on RGF BME gel at 4,800 cells/cm² (15,000 cells/well of 96-well plate) and incubated for 3 and 20 hours in the Endothelial Growth Medium-2 (EGM-2). **NOTE:** Use at least passage 2 cells for optimal tube assay results.
SVEC4-10 cells were seeded onto RGF BME gels or Normal BME gels at 4,800 cells/cm² on 96-well plates and incubated for 3 hours in the basal medium (EBM-2) or in the complete growth medium (EGM-2).

NOTE Tube formation requires angiogenesis stimulators (growth factors). The background tube formation is lower with RGF BME.
Induction of tube formation by growth factors and FBS

HUVECs were seeded on RGF BME gel (4,800 cells/cm²) and incubated for 6 hours in the Endothelial Basal Medium-2 (EBM-2) with and without FGF-2 and VEGF or in the Endothelial Growth Medium-2 containing 2% FBS, FGF-2, VEGF and other supplements (EGM-2). **NOTE the best tubes are with a combination of all the factors and serum.**
Endothelial cells do not form tubular-like network on thin BME gels

HUVECs were seeded onto RGF BME gels (4,800 cells/cm²) on 96-well plates and incubated for 6 hours in the Endothelial Growth Medium-2 (EGM-2). **NOTE:** At least 50 µl BME/well of 96 well plate required for optimal tube formation.
Summary

• Conditions of the cell culture are critical for successful results of the tube formation assay:
  • Let cells recover after freezing-thawing (do not use cells immediately after being thawed, passage cells once or twice)
  • Plate cells 24 hours prior to assay
  • Do not let cells grow more than 80% confluent
  • Do not use primary cell line beyond passage 10-12
• Chose extracellular matrix according to experiment design:
  • Normal BME promotes efficient tube formation by endothelial cells in the basal medium without additional angiogenic factors
  • Reduced Growth Factors (RGF) BME promotes a low level of tube formation in the basal medium
Summary (continued)

- Endothelial cells do not form tubes on the thin gels, therefore use appropriate volume of BME:
  - 50-80 µl of BME per well of 96-well plate
- Formation of the tubular-like network on BME gel depends on the seeding density of endothelial cells:
  - optimal seeding density is about 5,000 cells per cm²
- Length of the assay depends on the cell type:
  - primary endothelial cells develop tubular-like network in 6-20 hours.
  - immortalized cells will form tubes in 2-3 hours.

Ordering Information:
In vitro Angiogenesis Assay Kit (Tube Formation) Cat# 3470-096-K